

CRYPTOPAIN VACCINES, ANTIBODIES, PROTEINS, PEPTIDES, DNA AND
RNA FOR PROPHYLAXIS, TREATMENT AND DIAGNOSIS AND FOR
5 DETECTION OF *Cryptosporidium* SPECIES

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BACKGROUND OF THE INVENTION

Field of the Invention

This invention concerns vaccines, antibodies, proteins, 15 DNAs and RNAs for diagnosis, prophylaxis and treatment of *Cryptosporidium* species infections and for detection of *Cryptosporidium* species. In particular, this invention concerns *Cryptosporidium* species antigen comprised of a protein, as well as polyclonal and monoclonal antibodies 20 directed against the antigen, DNAs and RNA encoding the *Cryptosporidium* species antigen and fragments and analogs thereof, and methods for production of recombinant or fusion proteins. This invention also concerns methods for diagnosis, prophylaxis, treatment of *Cryptosporidium* infections and 25 detection of *Cryptosporidium* species.

BACKGROUND AND RELATED DISCLOSURES

The genus *Cryptosporidium* consists of Apicomplexan parasites that invade and develop within epithelial cells of the gastrointestinal, hepatobiliary and respiratory tracts of 30 a wide variety of vertebrates including reptiles, birds and mammals. *Cryptosporidium* was recognized as a cause of animal disease for several decades before the first cases of human cryptosporidiosis were reported in 1976. However, it was not

until 1982 that the magnitude of disease caused by this parasite in both AIDS patients and immunocompetent hosts began to be appreciated. Subsequently, *Cryptosporidium* has been found to be one of the most common causes of human diarrhea worldwide, and to be an increasingly recognized cause of diarrhea in children, animal care workers, and travelers. (*Cryptosporidium and Cryptosporidiosis in Humans*, Ed. Fayer, R., CRC Press, Boca Raton (1997)).

Large waterborne outbreaks of cryptosporidiosis caused by contaminated municipal water supplies in the US or in the UK have been noted in the last ten years (*N. Engl. J. Med.*, 320:1372 (1989), and 33:161 (1994)). The most recent outbreak in Milwaukee in April 1993 involved 400,000 persons and led to the subsequent deaths of more than 100 immunocompromised persons. Like a number of other waterborne outbreaks, the Milwaukee outbreak appears to have been due to contamination from farm or abattoir run-off and specifically to cryptosporidiosis among cows/calves. Nosocomial transmission in hospitals from patients to staff, patient to patient, and contaminated ice to patients and staff have also been well documented (*J. Infect. Dis.*, 158:647 (1985)).

Waterborne and nosocomial spread uncovered a number of biological characteristics of oocysts. First, the infectious dose of a parasite is very low. The ID₅₀ for human volunteers with normal immune systems is 132 oocysts *N. Engl. J. Med.*, 332:855 (1995). Second, infected hosts, for example calves, excrete large numbers of oocysts, on the order of 10¹⁰/day. Third, the oocysts are fully sporulated and ready to infect when excreted. Fourth, the oocysts are environmentally hardy. They remain infectious in cool, moist areas for 3-4 months. They are not killed by chlorine levels achievable in drinking water. Fifth, the oocysts are quite small, 4-6 μm , and are thus difficult to filter.

The clinical importance of cryptosporidiosis has increased markedly with the recognition of a life-threatening form of the disease in patients with immunodeficiency disorders such as AIDS, hypogammaglobulinaemia, and 5 chemotherapeutic immunosuppression. The prevalence of cryptosporidiosis in AIDS patients in the US is estimated to be 5-10% and in central Africa 40%. Immunodeficient patients may have fulminant cryptosporidial diarrhea that may persist until death, whereas the diarrhea of immunocompetent patients 10 is self-limited and rarely lasts more than 2-4 weeks. Cholera-like diarrhea is common in immunocompromised patients with reported losses of up to 17 liters per day. Hepatobiliary disease may result in severe abdominal pain and nausea. Removal of immunosuppression in chemotherapy patients 15 leads to resolution of the diarrhea. Some AIDS patients with cryptosporidiosis will be able to eliminate the parasite by induction of anti-retroviral therapy (Am. Intern. Med., 116:840 (1992)).

Among those who develop disease, a quarter have CD4 20 counts greater than 209, suggesting that the disease may occur relatively early in the course of HIV disease (Am. J. Epidemiol., 144:807 (1996)). Unfortunately, few details about the biology and molecular mediators of the disease process 25 have been described and so far no effective therapy has been discovered.

The infective forms of *Cryptosporidium*, called sporozoites and merozoites, appear to adhere to the host cell and release the contents of anterior organelles (rhoptries, micronemes or dense granules) during the invasion process 30 (Parasitol. Today, 8:28(1992)). Proteins involved in these events have in many instances been found to be the target of invasion blocking immunity *in vitro* and neutralization *in vivo* (Infect. Immun., 56:2538(1988)).

Active and passive immunization studies using malaria and Toxoplasma challenged or infected hosts have shown that certain secreted components of the apical complex organelles are the target of protective antibodies. In some cases, as 5 for example in the case of the circumsporozoite and merozoite surface proteins of malaria, these antigens are under development as vaccines.

While the actual interaction between *Cryptosporidium* and the host's immune system is poorly understood, it is known 10 that disruption of either the cellular or the humoral components can result in protracted cryptosporidiosis (Parasitol. Today, 8:24 (1992)). However, specific antibodies alone appear to be enough to neutralize the organism's 15 infectivity. *In vitro* and *in vivo* observations indicate that antibodies to *Cryptosporidium parvum* inhibit invasion and intracellular development leading to protection in challenge experiments, or amelioration of infection in established disease (Infect. Immun., 59:1172 (1991)).

One source of such antibodies is hyperimmune bovine 20 colostrum (HBC) collected from cows immunized with *Cryptosporidium* oocysts. Calves challenged with *Cryptosporidium* oocysts are protected from the development of disease by the administration of HBC (Infect. Immun., 61:4079 (1993)). Some immunocompromised AIDS patients infected with 25 *Cryptosporidium* have also responded to HBC with a reduction in or disappearance of the symptoms of the disease (Gastroenterology, 98:486 (1990)). Immunoglobulin from HBC (HBC Ig) has been found to inhibit the ability of the sporozoite to invade and/or develop intracellularly *in vitro* 30 and it has been used to immunoprecipitate at least 22 different surface radioiodinated proteins of *Cryptosporidium* sporozoites. Western blot analysis of proteins of whole oocysts which contain sporozoite, indicates that HBC

predominantly recognizes two proteins of sizes 250 Kd and >900 Kd (Infect. Immun., 61:4079 (1993)).

The use of HBC for human use is problematic. HBC produced using whole oocysts is batch dependent and this may 5 lead to the development of passive immune preparations which are not uniform in immunogenicity and potency. This generates a problem when these immune preparation are to be administered to human patients as such non-uniformity may result in failure of protection. In addition, it would be desirable to allow 10 preparation of large amounts of antigen expressed in heterologous systems than to purify oocyst.

Thus, there is a continuous need for immunogenic agents which are reasonably reproducible and have uniform and controllable immunogenicity and potency which agents would be 15 useful for the immunotherapy of cryptosporidiosis in both uncompromised and immunocompromised subjects, such as AIDS patients, and would allow the prophylaxis and treatment of cryptosporidiosis.

Additionally, there is a need to have available methods 20 for reproducible expression of specific target for *Cryptosporidium* antigen in large amounts, which antigen would provide a better immunogen. This approach requires that a specific *Cryptosporidium* antigen is cloned and identified as a potential candidate through its ability to elicit an 25 antibody response that is immunoprotective. Before antibodies produced in this manner are tested in or administered to humans or animals, testing in *in vitro* assay of their inhibitory effect on invasion or intracellular development of the *Cryptosporidium* organism in cultured cells and *in vivo* 30 studies would be desirable.

It is, therefore, a primary objective of this invention to provide *Cryptosporidium* cryptopain polyclonal or monoclonal antibodies and vaccines to be used for prophylaxis, treatment,

diagnosis and detections of cryptosporidiosis and to express a portion of the cryptopain sequence/locus to provide target protein antigens allowing production of recombinant anti-*Cryptosporidium* vaccines and passive immune products.

5 All patents, patent applications and publication cited herein are hereby incorporated by reference.

SUMMARY OF THE INVENTION

One aspect of this invention concerns vaccines, antigens, antibodies, proteins, DNAs and RNAs for prophylaxis, treatment 10 and detection or diagnosis of *Cryptosporidium* species or *Cryptosporidium* species infections.

Another aspect of this invention concerns a *Cryptosporidium* antigen protein comprising pre, pro, and mature enzyme sequences and their fragments.

15 Still another aspect of this invention concerns polyclonal or monoclonal antibodies directed against the *Cryptosporidium* antigen.

Still yet another aspect of this invention concerns a DNA and RNA encoding the *Cryptosporidium* antigen and fragments 20 thereof and the antigen pre, pro, and mature regions.

Another aspect of this invention concerns a polyclonal or monoclonal antibodies directed against invasive stages of Cryptosporidial species capable of preventing and ameliorating invasion of *Cryptosporidium* infection.

25 Still another aspect of this invention concerns a natural, synthetic or recombinant vaccine useful for active immunization of animals and humans against *Cryptosporidium* infection.

30 Still another aspect of this invention concerns a natural, synthetic or recombinant protein useful for preparation of passive immune products for treatment of established infection.

Another aspect of this invention concerns a natural, synthetic or recombinant DNA vaccine capable of endogenous production of inhibitory amount of anti-*Cryptosporidium parvum* antibodies.

5 Another aspect of this invention concerns a natural, synthetic or recombinant RNA vaccine capable of endogenous development of inhibitory amount of anti-*Cryptosporidium parvum* antibodies.

10 Still another aspect of the invention concerns a method for use of a pre pro enzyme portion of the cysteine proteinase molecule as a competitive inhibitor of the action of the mature enzyme.

15 Still yet another aspect of the invention is the use of antigen, antibody, DNA or RNA to detect the presence of the cysteine proteinase or antibodies to cysteine proteinase, or DNA or RNA encoding the cysteine proteinase, for diagnosis in a human or animal host or detection in the environment.

20 Another aspect of this invention concerns the sequence of a 401 amino acid protein comprising a cathepsin L-like cysteine proteinase of MW 45 kDa present in sporozoites and merozoites, and its amino acid and size variants including a deduced mature 226 amino acid protein of MW 25 kDa.

25 Another aspect of this invention concerns the DNA sequence of 1203 nucleotides encoding the 45 kDa protein, the cathepsin-like cysteine proteinase, cryptopain, its nucleotide and size variants and its upstream regulatory elements.

30 Another aspect of this invention concerns the RNA sequence determined by the DNA sequence of cryptopain and its nucleotide and size variants including polyadenylation sequence.

Still yet another aspect of this invention concerns a group of cryptopain recombinant or expressed protein targets of polyclonal antibodies which inhibit *Cryptosporidium*

infection, invasion, or adhesion.

Another aspect of this invention concerns a method for prophylaxis and treatment of *Cryptosporidium* or *Cryptosporidium* infections using vaccines, antibodies, 5 proteins, DNAs and RNAs of the invention.

Still yet another aspect of this invention concerns a method of prophylaxis, treatment, inhibition or retardation of a *Cryptosporidium* infection comprising administering to a subject in need of such treatment an amount of an 10 anti-*Cryptosporidium* polyclonal or monoclonal antibodies prophylactically or therapeutically effective to provide immunity against infection or treatment for disease.

Still yet another aspect of this invention concerns a method of prophylaxis, treatment, retardation, or inhibition 15 of *Cryptosporidium* infection comprising administering to a subject in need of such treatment a vaccine comprising the polypeptide of this invention or its DNA or RNA capable of endogenous stimulation of the production of inhibitory amount of anti-*Cryptosporidium* antibodies or protective cellular 20 immune responses.

Still yet another aspect of this invention concerns a method for diagnosing *Cryptosporidium* infection of a subject, comprising steps:

(a) contacting a body specimen, fluid or tissue obtained 25 from the subject with an anti-*Cryptosporidium* monoclonal or polyclonal antibody; and

(b) detecting the formation of antibody-antigen complex wherein the presence of the complex indicates the presence of a *Cryptosporidium* organism in the subject.

30 Still yet another aspect of this invention concerns a method for detecting anti-*Cryptosporidium* antibody in a subject, said method comprising steps:

(a) contacting a body specimen, fluid or tissue obtained from the subject with the cryptopain; and

(b) detecting a formation of antibody-antigen complex wherein the presence of the complex indicates the presence of 5 a *Cryptosporidium* antibody in the subject.

Still another aspect of this invention is a *Cryptosporidium* diagnostic or detection kit comprising anti-*Cryptosporidium* specific monoclonal and polyclonal antibodies or antigen according to the invention and a means 10 for detection of an antibody-antigen complex.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram showing the strategy model for developing a probe for the *Cryptosporidium* cysteine proteinase using consensus oligonucleotide primers for PCR amplification 15 of genomic DNA. The model is compared to previously published diagrams of the primary structure of cysteine proteinases from other organisms.

Figure 2 is the DNA sequence of cryptopain (SEQ ID NO: 1) comprising sequences encoding segments for the pre and pro 20 regions (SEQ ID NO: 2), mature enzyme coding region (SEQ ID NO: 3) and 3' and 5' flanking sequences.

Figure 3 is the protein sequence of cryptopain (SEQ ID NO: 4) comprising segments for the pre and pro regions (SEQ ID NO: 5) and for mature enzyme (SEQ ID NO: 6).

25 Figure 4 is an amino acid alignment showing marked amino acid similarities of cryptopain to other cathepsin-like cysteine proteinases (SEQ ID NOs: 4, 7 and 8).

Figure 5 shows a genomic Southern analysis of *Cryptosporidium* DNA using the cryptopain probe.

30 Figure 6 shows a Kyte Doolittle hydropathy plot indicating an N-terminal hydrophobic sequence consistent with membrane targeting and secretion of cryptopain.

Figure 7 are oligonucleotide sequences used to generate

DNA fragments of the cryptopain gene. Figure 7A1 is a degenerate primer based on the conserved cysteine (sense) and Figure 7A2 is a degenerate primer based on conserved arginine (antisense) of the *P. vinckeii* cysteine proteinase gene. These 5 primers were used to amplify the 459 bp fragment of cryptopain from *C. parvum* DNA. Figure 7B shows primers used to directionally clone the entire *C. parvum* gene comprising pre, pro and mature protein encoding regions, to be expressed as a thioredoxin fusion protein. Figure 7B1 is the sense and 10 Figure 7B2 is the antisense oligonucleotide.

Figure 8 is a diagram of pTrxFus showing the directional cloning strategy.

Figure 9 is a Western blot of cryptopain expressed as a thioredoxin fusion protein and detected by anti-thioredoxin 15 antibody.

Figure 10 are graphs showing percentage of invasion/intracellular development of *Cryptosporidium parvum* sporozoites *in vitro* in MDCK cells in the presence of inhibitors of cysteine proteinases. Figure 10A is a 20 comparative graph of three cysteine proteinase inhibitors biotinylated fluoromethylketone (BPAFMK) (Figure 10B); trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane E64 (Figure 10C); and proprietary compound K-111 (Figure 10D). Figures 10B-10D show standard deviations.

25

DEFINITIONS

As used herein:

"Cryptopain" or "*Cryptosporidium* antigen" means a protein which is a cathepsin L-like cysteine proteinase having a function in invasion and infection of host cells by 30 *Cryptosporidium*. Cryptopain is represented by a protein containing 401 amino acids and is identified as SEQ ID NO: 4 (Figure 3) comprising a protein of MR 45 kDa. Homology to other cathepsin L-like cysteine proteinases seen in Figure 4

indicates that the mature active enzyme is cleaved after amino acid 175 one residue N-terminal to a conserved prolines and comprises a 25 kDa protein of 226 amino acids. Cryptopain also includes size and sequence variance proteins which 5 maintain the same function.

The "structure" or "structural characteristics" of cryptopain defines a protein, and DNA and RNA encoding the cryptopain protein and includes all structural variations, mutations and fragments exhibiting the same function.

10 The "functionality" or "functional characteristics" of cryptopain is defined by the action of the protein and structural variants described, such that infection and disease occurs.

15 "Inactive enzyme" means enzyme comprised either of mature enzyme regions and pro regions, or mature enzyme and pro and pre regions wherein the pro or pre pro regions are responsible for the mature enzyme nonfunctionality or for the inhibition of its function.

20 "Active enzyme" or "mature enzyme" means functional enzyme and is comprised of the mature region. Mature enzyme contains the catalytic active sites of the cysteine proteinase and typically begins with one residue N-terminal to a conserved proline.

25 "Pro" or "pro region" means the contiguous amino acid sequence which renders the mature enzyme inactive by its structural association with it.

30 "pre" or "pre region" means the terminal amino acid sequence which is contiguous with the pro region and may contain a signal for trafficking movement of inactive enzyme in the cell.

"The gene" or "genes encoding cryptopain" means DNA encoding the cryptopain protein.

"Sporozoites or merozoites" mean any life stage which

may invade or develop in the host cells and any variant or mutant of said life stages.

5 "Antibodies" means proteins which structurally interact with the target antigen and are produced when the antigen is introduced into an animal, such that they stimulate the immune system. The term also includes antibodies produced *in vitro*, such as chimeras, or hybridoma cell cultures, as well as hybridomas or chimeric constructs introduced into a host to provide an *in vivo* antibody.

10 "Antibodies to cryptopain" means proteins which structurally interact with the target antigen cryptopain and inhibit invasion, infection or development of the sporozoites or merozoites in the host cell.

15 "Monoclonal antibodies" means the monovalent antibodies produced by an B cell fused to an immortalized cell producing specific antibody to cryptopain.

"Polyclonal antibodies" means antibodies directed at cryptopain which are not monovalent and are the products of multiple B cells in character.

20 "Cryptosporidium antigen" means a protein with or without carbohydrate attached thereto which defines a capacity of *Cryptosporidium* sporozoites and merozoites to infect and develop in host cells.

25 "Cryptopain DNA" means the sequence of 1203 polydeoxyribo nucleotides identified in SEQ ID NO: 1 (Figure 2) which encodes the amino acid sequence of *Cryptosporidium* antigen (SEQ ID NO: 4) and any variants, mutations and fragments thereof which correspond to or would detect genes encoding the antigen and includes specific PCR oligonucleotide primers for 30 amplification of cryptopain sequences and fragments of sequence used as genetic probes for detection of cryptopain sequence. Also included is DNA inserted into host cells for the purpose of *in vivo* expression of target antigen in order

to stimulate the host immune system.

"Cryptopain RNA" means the sequence of 1203 nucleotides which encodes the protein sequence of cryptopain protein (SEQ ID NO: 4) (Figure 3) and any variants, mutations and fragments thereof including polyadenylation tail which correspond to or would detect genes encoding the antigen. RNA probes and RNA inserted into host cells for the purpose of *in vivo* expression of target antigen in order to stimulate the host immune system are included.

"Vaccine" means protein, recombinant protein, DNA or RNA from cryptopain which, upon introduction into a host, is able to provoke an immune response including but not limited to the production of antibodies, cytokines and other cellular responses.

"Detection" means establishing or providing evidence for the presence or prior presence of living or dead *Cryptosporidium* by detecting cryptopain protein, *Cryptosporidium* protein specific activity, DNA or RNA in the host, in a host tissue specimens, or in environmental samples including water, soil, food, etc.

"Diagnosis" means establishment of the presence or prior presence of *Cryptosporidium* infection or disease by using the cryptopain protein, *Cryptosporidium* protein specific activity, DNA or RNA as a component of a diagnostic assay according to the invention.

"Prevention or prophylaxis" means the immunization or vaccination of the host with a vaccine of the invention such that *Cryptosporidium* disease or infection does not occur.

"Treatment" means therapeutic use of any protein or antibody to inhibit *Cryptosporidium* infection in a host.

"Host" or "subject" means human, or animal including birds and cattle.

"Regulatory elements" means nucleotide sequences which

control the expression of genes they regulate, typically by interaction with other macromolecular species such as protein.

"Active immunity to infection" means ability of the organism to produce specific responses such as production of 5 cytokines, lymphokines, antibodies or other substances, or cellular capacity to inhibit or retard infection in response to a contact with antigen.

"Passive immunity to infection" means the transfer to a host of the specific antibodies or other substances or cells 10 capable of inhibiting or retarding infection.

"*Cryptosporidium* species" means any organism belonging to the genus *Cryptosporidium*, such as, for example, *Cryptosporidium parvum* or *Cryptosporidium muris*, but also includes currently less well characterized other organisms 15 such as, for example, Cyclospora and similar organisms, such as *Eimeria*. *Cryptosporidium* species comprise Apicomplexan parasites which primarily invade cells of gastrointestinal tract and cause disease in a susceptible host.

"Recombinant vaccines" means DNA/RNA/protein segments 20 propagated or expressed in foreign system. This includes all vaccines other than biologically derived vaccines.

"Biologically derived vaccines" means vaccines made from a protein or carbohydrate generated in the organism of origin.

DETAILED DESCRIPTION OF THE INVENTION

25 The current invention is based on findings that cryptopain, a cathepsin L-like cysteine proteinase, localized at the *Cryptosporidium* sporozoites surface or within its cell, is involved in *Cryptosporidium* infectivity and that such infectivity can be prevented by cryptopain inhibitors.

30 Cryptopain deduced amino acid sequence shows homology to other cathepsin L-like cysteine proteinases indicating that the mature active enzyme is a 25 kDa protein of 225 amino acids. Cryptopain DNA has been isolated, purified, sequenced

and recombinantly produced. Cryptopain fusion protein in which the fusion partner is thioredoxin has also been recombinantly produced.

Due to its unique biological activity, cryptopain may be 5 advantageously used for prophylactic, therapeutic, diagnostic and detection purposes.

This invention, therefore, relates to isolated native and recombinantly produced cryptopain; cryptopain amino acid, DNA and RNA sequences; and to vaccines, antibodies, proteins and 10 synthetic proteins, DNAs and RNAs useful for prophylaxis, treatment, diagnosis and detection of infections caused by any *Cryptosporidium* organism or any organism belonging to *Cryptosporidium* species.

More specifically, the invention concerns identification 15 and cryptopain of a *Cryptosporidium* antigen, comprised of a protein or polypeptide, identification of DNA of the *Cryptosporidium* antigen gene within the locus, sequencing DNA encoding the *Cryptosporidium* antigen, expressing portions of the locus encoding the *Cryptosporidium* antigen and using the 20 expressed antigens for preparation of vaccines or for preparation of polyclonal or monoclonal antibodies.

I. Cryptopain - Cryptosporidium Parvum Antigen

Cryptopain is cathepsin L-like cysteine proteinase. It is structurally and functionally similar to other cysteine 25 proteinases, represented, for example, by *Carica papain* and *Plasmodium vinckeii* cysteine proteinase, and its activity is inhibited by group of cysteine proteinase specific inhibitors.

A. Cysteine Proteinases - Their Function, Structure and Inhibition

30 There are four major classes of proteinases for which the catalytic mechanism has been defined. These proteinases are designated cysteine, aspartic, metallo and serine proteinases. The major mammalian cysteine proteinases are the lysosomal

cysteine proteinases, cathepsins B, H and L proteinases and the cytoplasmic calpains. Mammalian cysteine proteinases B and L are also active at neutral pH, and are found outside the cell and may function in the degradation of extracellular 5 proteins. The sequences of the protozoan cysteine proteinases identified to date show that they are more closely related to cathepsin L than to cathepsin B. Cysteine proteinases essentially contain amino acids cysteine, histidine and asparagine which are important for the action of the 10 proteinases. The sulfonium ion of the cysteine provides the nucleophilic attack on the carbonyl group of the targeted peptide bond in order to effect hydrolysis of the bond.

Calpains and cathepsins are generally distinguished from each other by their cellular locations and by their inhibition 15 profile. For example, cathepsins, but not calpains, are inhibited by the peptidyl diazomethane and peptidyl fluoromethylketone inhibitors Z-phe-ala-CHN₂ (diazomethane) and Z-phe-ala-FMK (fluoromethylketone). Both lysosomal cysteine proteinases and calpains are inhibited by the class-20 specific inhibitor E64 and the more general inhibitor leupeptin.

Peptide inhibitors have been used to determine the peptide bond specificity of proteinases. The specificity of the inhibitor is determined by the amino acid residues, for 25 example, phe-ala residues, which bind in the pocket formed by the active sites of the enzyme. Peptide inhibitors only bind to active enzyme, i.e. enzyme which has a conformationally correct enzyme pocket. Peptide inhibitors are useful for detection of the presence of specific types of cysteine 30 proteinases in living systems as they may allow the localization or detection of enzymatic activity in the absence of isolation and purification of the enzyme with the subsequent development of antibody probes. Since isolation of

active enzyme by biochemical techniques requires large amounts of material and the isolated enzyme is often not stable, use of peptide inhibitors instead is very advantageous.

5 Proteinase inhibitors are a new type of agent for treatment of protozoan infection. Cloning of genes for selected proteinases, expression of the proteinases, and molecular modeling of the proteinases are techniques which have facilitated the development of cysteine proteinases inhibitors specific for a given enzyme, such as for example, 10 falcipain of *P. falciparum*. In addition, the differences between mammalian and protozoan cysteine proteinases and between cysteine proteinases of specific protozoa allow development of detection techniques for the organism based on the acting of the enzyme, DNA, RNA and antibodies.

15 B. Cryptopain Gene Cloning, Sequencing and Genomic Southern Analysis

In order to provide consistently the same antigen for production of antibodies or vaccines, and for recombinant production of fusion proteins and other agents useful for 20 prophylactic therapeutic and diagnostic purposes, cryptopain was cloned, sequenced and genomic Southern analysis was performed to determine whether there was one or more cysteine proteinase similar to cryptopain.

Degenerative oligonucleotides were synthesized from the 25 sequences encoding the active sites of papain like cysteine proteinases centered around the active site cysteine and histidine as seen in Figure 1 and around the active site arginine described in Example 2. In Figure 1, the primary structures of cysteine proteinases for *L. mexicana*, *T. brucei*, 30 and human cathepsin-L are compared to the primary structure of *C. parvum* cryptopain. The diagram in Figure 1 shows the conserved cysteine and histidine residues involved in the active site, and the cysteine residues apparently involved in

disulfide bridges. For cryptopain, the conserved cysteine is C-24, the conserved histidine is H-164. The proposed disulfide bridges are 21-65, 56-103 and 158-210. Figure 1 is a modified Figure 19.4, from Biochemical Protozoology, 214, 5 Ed. G. Coombs, et al., Tayla and Francis, London (1991).

For fragment amplification, a number of oligonucleotides were tried without success until oligonucleotides specific for the *Plasmodium vinckeii* cysteine proteinase, described in Example 2 were identified. These oligonucleotides were found 10 to be suitable for and were therefore used to amplify a fragment of genomic DNA from Iowa isolate *Cryptosporidium parvum* oocysts.

The fragment was sequenced using methods described below and known in the art and found to encode a 459 bp portion of 15 a cysteine proteinase gene seen in Figure 2, DNA residues 869-1326. The fragment was hybridized to an Iowa isolate genomic Southern blot which indicated that the cysteine proteinase was a single copy gene. Results are seen in Figure 5.

Figure 5 is a genomic Southern analysis of 20 *Cryptosporidium* DNA using the cryptopain probe. In Figure 5, lane 1, the probe hybridizes to two Hind III fragments. These fragments are of approximate size 1.5 and 4 kb. In lane 2, the probe hybridizes with a Hae III fragment of 1.2 kb. In lane III the probe hybridizes to fragments of 1.2 and 4 kb of 25 a Hind III/Hae III digest. In lane 4, the probe identifies fragments of 10 and 1 kb in an NsiI digest. In lane 5, the probe identifies a single band of 4 kb in an ScrII digest and in lane 6 it identifies fragments of 1.0, .5 and 4 kb in an NsiI/ScrII digests. The presence of 1 or 2 bands greater than 30 the size of the probe in all digests indicates that the cysteine proteinase is a single copy gene.

The 459 bp Iowa fragment was then used to identify naturally infected neonatal calf (NINC) according to Infect.

Immun., 61:40 (1993) library clone which encoded the entire gene and 5' and 3' flanking regions. The sequence of this clone appears in Figure 2 and is identified as (SEQ ID NO: 1). The sequence of the open reading frame was determined.

5 The corresponding sequences of the NINC clone and the 459 bp sequence of the Iowa cysteine proteinase isolate are identical indicating that cryptopain is highly conserved in these isolates and that its function is essential for *Cryptosporidium*.

10 Sequences identified as SEQ ID NOS: 1-6 disclosed in this invention are new. These sequences represent nucleotides and amino acid sequences of *C. parvum* antigen. They were prepared according to methods described in Examples 1, 2 and 3.

15 SEQ ID NO: 1 is the DNA sequence of the *Cryptosporidium* cryptopain. The sequence (SEQ ID NO: 1) comprises 1663 base pairs and comprises 5' and 3' flanking sequences, pre, pro (SEQ ID NO: 2) and mature enzyme (SEQ ID NO: 3) sequences.

20 SEQ ID NO: 4 is the amino acid sequence of the cryptopain. The cryptopain contains 401 amino acids and contains pre and pro fragments (SEQ ID NO: 5), and mature enzyme (SEQ ID NO: 6).

25 Sequences 7-8 are known and correspond to cysteine proteinases isolated from other organisms, namely from *Carica* and *P. vinckeii*. Homology between these and the current *C. parvum* cysteine proteinase is shown and described in Figures 1 and 4.

Sequences identified as SEQ ID NOS: 9-12 are primer sequences.

30 Sequences SEQ ID NOS: 13-15 represent amino acid fragments of cryptopain.

Sequence SEQ ID NO: 16 represents a 1206 fragment of cryptopain DNA.

C. Structure of the Cryptopain Gene and Its Encoded

Protein

5 The function of cryptopain is highly correlated with the structure of the protein which is determined by the corresponding sequence. In addition, regulation of the function is, at least in part, dependent upon the presence of the pro sequence.

10 Sequence identified as SEQ ID NO: 1 (Figure 2) is a DNA sequence of cryptopain. Sequence identified as SEQ ID NO: 4 (Figure 3) is its corresponding protein. Search of the Gene Bank and Swiss Protein Bank revealed that these sequences were highly homologous to cathepsin L- like sequences of various organisms as seen in Figure 4.

15 Figure 4 is an amino acid alignment showing marked amino acid similarities of cryptopain of *Cryptosporidium* (SEQ ID NO: 4) cysteine proteinase (papain) of *Carica* (SEQ ID NO: 7) and mature cysteine proteinase *Plasmodium vinckeii* (SEQ ID NO: 8). In Figure 4, the mature enzyme of *P. vinckeii* and the pre pro enzymes of cryptopain and papain (*Carica*) are lined up.

20 The active site cysteine shown at site 200 is embedded in a 7 amino acid fragment CGSCWAF (SEQ ID NO: 13) which is conserved in all three enzymes and was one of the sites chosen to make degenerate oligonucleotides primers listed in Figure 7A. There is not a high degree of conservation of sequence between the 3 enzymes around the active site histidine seen at 25 341. However, the conserved arginine at 392 is embedded in an amino acid fragment YWL/IVRNSW (SEQ ID NO: 14) which only differs by 1 amino acid in *P. vinckeii* cysteine proteinase and cryptopain. This substitution of I and L was not engineered into the degeneracy of the *P. vinckeii* oligonucleotide. 30 Nonetheless, the degenerate oligonucleotide 782 containing sequence for VRNFW (SEQ ID NO: 15) and the active site cysteine oligonucleotide 781 were specific enough to amplify the 459 bp fragment. Unlike cryptopain, the *P. vinckeii* has a

large insertion seen in amino acids 358-386 between the conserved cysteine and arginine that were the basis for nucleotide PCR of the 459 bp *C. parvum* fragment.

D. Production of Cryptopain Recombinant Protein

5 Recombinant cryptopain proteins are useful as antigens for preparing antibodies which will inactivate cysteine proteinase and provide antibody probes to detect the presence of the organism in the environmental and clinical diagnostic setting. Their recombinant production is therefore important.

10 Recombinant proteins of the invention were produced as described in Example 5. Generally, the 1203 bp cryptopain open reading frame (ORF) is engineered for in frame expression as a thioredoxin fusion protein in the Invitrogen vector pTrxFus, or any other suitable vector seen diagrammed in 15 Figure 8. This vector is used to create C-terminal fusions to *E. coli* thioredoxin. There is a multiple cloning site which allows in frame fusion of foreign protein with thioredoxin. Between the thioredoxin and the foreign protein there is an enterokinase cleavage site. Enterokinase treatment permits 20 the release of thioredoxin from the protein. pTrxFus DNA is digested with for example KpNI and XbaI and the intervening fragment is removed for example, by gel purification.

Primers 7B1 and 7B2 were used to amplify the pre pro 25 enzyme sequence from Iowa *Cryptosporidium* DNA. The primer 7B1 has a KpN1 site and the primer 7B2 has an XbaI site engineered into the 5' end of the oligonucleotides. These enzymes are used to digest the amplified DNA so that it could be inserted directionally and in frame into the KpnI/XbaI restriction 30 digested pTrxFus. Then, the vector, such as pTrxFus, containing the sequence for the pre pro enzyme, is used to transform competent *E. coli* cells. Ampicillin resistant transformants are then analyzed for plasmid DNA by restriction with KpNI-XbaI and by sequence for the presence, orientation

and reading frame of the gene. Clones containing the same gene are induced for expression of cryptopain and expression of the fusion protein, such as for example cryptopain-thioredoxin, at 57 kD, was analyzed by SDS-PAGE as seen in 5 Figure 9, followed by immunoblot with antithioredoxin antibody. Conditions for optimal production of soluble protein in *E. coli* are assessed.

Results of the actual preparation of recombinant cryptopain using vector pTrxFus are seen in Figure 9. Figure 10 9 shows proteins harvested from a lysed cell culture, i.e., the soluble supernatant proteins. Lane 1 is wild type thioredoxin. Lanes 2, 3 and 4 are thioredoxin cryptopains harvested from cell culture at 2, 3 and 4 hours of growth of thioredoxin cryptopain. The pellet fraction showed no fusion 15 protein indicating that the cryptopain-thioredoxin is wholly soluble. Growth was maximal at 3 hours and degradation products of Mr less than 57 were visible at 4 hours indicating that the optional time for harvesting culture was around 3 hours.

20 Fusion protein may be purified by osmotic shock or heat treatment of cell lysates to produce highly purified fusion protein. The fusion protein is advantageously cleaved with enterokinase at a cleavage site comprising 4 asparagine and 1 lysine sequence.

25 Production of cryptopain may be accomplished in multiple prokaryotic or eukaryotic cells, including baculovirus, insect cells, yeast and mammalian cells. Cryptopain is purified by any suitable method known in the art, such as incorporation of histidine and purification by nickel chromatography, heat 30 treatment of fluoredoxin fusion protein with subsequent harvesting of soluble protein.

I. Inhibition of Sporozoite Invasion

In order to determine whether the invasion of

Cryptosporidium sporozoites may be inhibited, active site inhibitors of cathepsin L-like cysteine proteinases were investigated.

5 Cryptosporidiosis infection is caused by the invasion of cells with *Cryptosporidium*, typically *Cryptosporidium parvum*. In order to provide prophylactic, therapeutic, diagnostic or detection agents, it is necessary to determine what the function of cryptopain in the process of cell invasion is, and whether or not during the *Cryptosporidium* cell invasion 10 cryptopain acts at the surface of the sporozoites. For this reason, studies were performed using known inhibitors to determine entry of the sporozoites into the cells.

Because the sequence of cryptopain (SEQ ID NO: 4) had high homology with other cysteine proteinases, and has an N-15 terminal hydrophobic region, it was decided to determine if *C. parvum* secreted cysteine proteinase. The biotin modification of phe-ala-fluoromethylketone (BPAFMK) makes it unable to enter intact cells. Therefore, the biotinylated phe-ala 20 fluoromethylketone was used to determine whether a cathepsin-L like cysteine proteinase was active either at the surface of the *Cryptosporidium* sporozoites or in the supernatant media during invasion of Madin Darby canine kidney (MDCK) host cells and whether it allows *C. parvum* to enter the cells. Results are seen in Figure 10.

25 Figure 10A shows the % of sporozoites invasion as a function of the concentration of three cysteine proteinase inhibitors, namely inhibitors trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E64), obtained from Sigma, St. Louis, BPAFMK, obtained from Enzyme Systems, Dublin, 30 California and K-III, research drug of Arris Pharmaceutical, South San Francisco, California. The inhibitors were administered within the range of 10^{-3} to 10^1 (0.001 to 10 mM).

As seen in Figure 10, one hundred nM of all three

inhibitors decreased invasion by *Cryptosporidium* to 20-30% of untreated controls. The inhibition of invasion of sporozoites by BPAFMK (Figure 10) shows that cryptopain is involved in a proteolytic events which are necessary for invasion and 5 intracellular development of *Cryptosporidium*. Results seen in Figure 10 therefore show that cryptopain is either localized at the surface of the *Cryptosporidium* sporozoites, is a part of the sporozoites membrane or is localized internally and is released during the invasion of the host cell.

10 Assessment of other studied cysteine proteinase inhibitors (E64 and K-III) which were not chemically modified to prevent entry into the cell indicate that there is more than one cathepsin-L-like cysteine proteinase inhibitor which will prevent invasion and intracellular development.

15 Although not listed here, it is to be understood that other cysteine proteinase inhibitors, as long as these inhibitors inhibit *Cryptosporidium* invasion, are intended to be within the scope of this invention. The examples of active site inhibitors are trans-epoxysuccinyl-L-leucylamido-(4-20 quanidino) butane (E64), fluoromethylketone, diazomethanes, vinyl sulfones and cystatins.

Another class of inhibitors derived from pro region of cryptopain and its derivatives change the active enzyme into a proenzyme.

25 As described above, the complete DNA and amino acids structures of cryptopain (SEQ ID NOS: 1 and 4) comprise pre, pro and mature enzyme (SEQ ID Nos: 2 and 5) sequences (SEQ ID NOS: 3 and 6), delineated in Figure 1 and in Figure 4. These pre, pro and mature regions or elements are identified on the 30 basis of homology to previously discovered and investigated cysteine proteinases, seen in Figure 1, compared to *Cryptosporidium parvum* cryptopain. Biochemical Protozoology, (supra). The N-terminus of the cryptopain sequence contains

pre pro sequence (SEQ ID NO: 2). The cryptopain pre sequence is predicted by Kyto-Doolittle hydropathy plot seen in Figure 6 to contain a hydrophobic sequence. Such sequences typically target the protein to a membrane.

5 Inactive cysteine proteinases are called proenzymes. Proenzymes of cysteine proteinases consist of at least a pro amino acid sequence which interacts conformationally with the contiguous mature enzyme sequence to render it inactive until the pro sequence is cleaved releasing the active mature 10 enzyme. Recent evidence indicates that the pro sequences of cysteine proteinases are excellent specific inhibitors of their respective mature active enzymes (Protein Eng., 8:59 (1995)).

15 Thus, the pro sequence (SEQ ID NO: 5) of cryptopain is a good candidate and may be produced by recombinant or synthetic means for use as a pharmacological agent to prevent *Cryptosporidium* infection and/or the consequences of 20 infection.

25 III. Prophylaxis or Treatment via Passive or Active Immunization

For protection and treatment of human or animal subjects subjected to exposure to *Cryptosporidium*, or subjects already suffering from *Cryptosporidium* infection, both passive or active immunization using the cryptopain antigen is 25 appropriate.

Surface active enzymes with confirmed essential functions for the parasite infectivity, like cryptopain, are targets for passive or active immunization. Cryptopain binds to antibodies which specifically bind to epitopes of 30 *Cryptosporidium* which are recognized by B and T cells.

For prophylactic, therapeutic or diagnostic purposes, the proteins of the invention are produced in large amounts by inserting the *Cryptosporidium parvum* DNA, described above,

into an expression vector such as pGEX, pET-9d, pTrxFus or baculovirus obtained from Invitrogen. The thus constructed hybrid vector is then used to transform or transfect a host. The host cells carrying the hybrid vector are then grown in a 5 nutrient medium to allow the production of the gene product.

A number of transfer vectors are available for the production of protein from both full length and partial cDNA and genomic clones. Fused or non-fused protein products, depending on the vector used, constitute up to 50% of the 10 total protein produced in infected cells. The thus obtained recombinant proteins are frequently immunologically and functionally similar to the corresponding endogenous proteins.

The obtained polypeptide is purified by methods known in the art or described in Examples. The degree of purification 15 varies depending on the use of the polypeptide. For use in eliciting polyclonal antibodies, the degree of purity may not need to be very high. However, as in some cases impurities may cause adverse reactions, purity of 90-95% is typically preferred and in some instances even required. For the 20 preparation of a pharmaceutical composition, however, the degree of purity must be high, as is known in the art.

When in a therapeutic composition, the polypeptide is combined with appropriate pharmaceutically acceptable 25 excipients adjuvants and used for the immunization of immunocompetent patients who are at risk for cryptosporidiosis either at the time of immunization or in the future.

This group includes, but is not restricted to, HIV positive individuals who are still able to respond to vaccination, animal workers, health care workers, day care 30 center children and their caretakers, and children in the developing world.

A. Antibodies and Their Production

A polyclonal or monoclonal antibody to native or

recombinant protein of the invention are useful in diagnosing and detecting *Cryptosporidium* as well as for treatment by providing a protection against the *Cryptosporidium* infections.

Anti-*Cryptosporidium* polyclonal antibodies recognizing the cloned polypeptide are preferred over a monoclonal antibody (MAb) because they recognize multiple epitopes on the target polypeptide.

According to the method of the current invention, large amounts of recombinant cryptopain are produced by scale up processes in commercial plants which enables production of a corresponding large quantity of polyclonal antibodies^{and} ~~or~~ ^{and} ~~or~~ ~~or~~ immunogen for active immunization. The antibodies to recombinant expressed protein can also be produced according to the invention using the standard method available for production of the antibodies to native protein.

Cryptopain comprising epitopes of *Cryptosporidium* that is recognized by intact B and/or T cells is produced in large amounts as described above and in Examples, purified and used to detect or characterize anti-*Cryptosporidium parvum* antibody in the body substances of populations at risk of prior or current cryptosporidial infection. Cryptopain is also used for immunization. Typical intramuscular immunization schedules are as follows.

Cryptopain plus equal volume complete pharmaceutically acceptable adjuvants and excipients is used at the beginning of immunization. Antigen plus equal volume incomplete adjuvant is used at week 2. Antigen plus equal volume incomplete adjuvant at week 4.

In addition, antibodies to such antigens are obtained by immunizing animals, such as rabbits or goats, with the polypeptide plus adjuvant, as described above.

The antibodies of the invention are also used to detect *Cryptosporidium* antigens in body substances, for example,

stools of populations at risk of cryptosporidial infection by, e.g., collecting stool samples (Manual of Clinical Microbiology, 1986, supra), mixing with Streather's solution 1:4, and incubating with antibody followed by addition of a 5 fluorescein conjugated second antibody. In alternative, colorimetric labeling which do not require special microscope equipment or other detection methods also suitable.

B. Biologically Derived or Recombinant Anti-Cryptosporidium Vaccines

10 Vaccine is a biologically derived or recombinantly prepared agent useful for artificially acquired immunization in a host. The current invention describes a production and provides biologically derived and recombinant vaccines for active immunization of animals and humans against 15 cryptosporidiosis and for the preparation of passive immune products for treatment of the established infection.

The scope of the invention is, therefore, intended to include both biologically derived or recombinantly prepared vaccines based on the antigens of the invention.

20 A recombinant vaccine is produced by identifying the relevant antigen or antigens of *Cryptosporidium* species, cloning them and expressing them using suitable vectors. This approach yields immunogens which are reproducible in sufficiently large quantities to allow preparation of vaccine 25 for active immunization. Recombinant vaccines are useful for immunization of the potential *Cryptosporidium* host, such as for example for inoculation of cows, and to produce the host's own antibodies against *Cryptosporidium* infection.

30 Additionally, the recombinant vaccines may be used for production of passive immunotherapeutic agents. For example, when the cow is inoculated with the vaccine it begins to produce hyperimmune colostrum. Hyperimmune colostrum from the immunized cows is then purified to yield Ig for passive

immunotherapy of immunocompromised persons, primarily AIDS patients, children, etc.

These vaccines are also useful for widespread use in calves to provide a primary protection against *Cryptosporidium* infection. Providing the herd with anti-*Cryptosporidium* immunity decreases the risk for waterborne outbreaks of cryptosporidiosis in areas where the watershed includes dairy lands. This provides a secondary benefit to human residents of those areas.

10 In addition, DNA or RNA may be introduced into a host such that propagation and/or expression of the encoded protein occurs in the host utilizing a so called "foreign expression system".

15 Anti-*Cryptosporidium* vaccine of the invention contains a *cryptosporidium* antigen identified by the invention, modified in such a way that it is incapable of producing the *Cryptosporidium* symptoms but at the same time it is capable of eliciting the production of specific protective antibodies against the disease when introduced in the body.

20 Protection from cryptosporidiosis appears to be due to mucosal immunity which, if absent in AIDS patients, is difficult to establish but, if present, may afford protection against clinical cryptosporidiosis as AIDS progresses.

25 Thus, the invention describes vaccines able to provide active B cell-immunity and potentially T cell immunity against cryptosporidiosis in normal persons, in persons at risk for AIDS or in otherwise immunocompromised patients.

C. DNA and RNA Vaccines

30 Recently, new approaches appeared which utilize so called DNA or RNA vaccines. These approaches are described in Science, 259:1745 (1993), hereby incorporated by reference.

DNA or RNA vaccines or native immunity are produced according to the methods described *Ibid*. Briefly, nucleic

acid vectors containing *Cryptosporidium* antigen DNA nucleic acid are injected, preferably intramuscularly to the host. The nucleic acid enters or is transmitted where it results in production of antigen. The antigen elicits immune responses 5 in the form of specific anti-*Cryptosporidium* antigen antibody or cell mediated immune events. In this way, the host receives DNA or RNA and provides his/her own humoral immunity and/or cell mediated responses.

IV. Diagnostic/Detection Utility

10 An important part of this invention is a method of diagnosing *Cryptosporidium* infection or detection of *Cryptosporidium* in the tissue samples or in the environment.

The diagnostic method comprises contacting a body fluid or tissue with an anti-*Cryptosporidium* polyclonal or 15 monoclonal antibody having specificity for the antigen of this invention or its fragments, or vice versa, and ability to detect any selective binding of the antibody to any antigenic *Cryptosporidium* proteins present in the body fluid, tissue or specimen or selective binding of the antigen to the anti-20 *Cryptosporidium* antibody.

The detection of the antibody-antigen complex in body specimens or environmental samples may be conducted by any method known in the art. The detection methods include solid phase, double antibody, sandwich double antibody, and triple 25 antibody assays, including ELISA and the like. Also suitable are enzyme-linked immunoassays and radioactively labeled assays.

Examples of body specimens are stools and other liquid or solid body output or tissue samples obtained from a subject. 30 Examples of body fluids are blood, serum, saliva, urine, and the like. Methods for the preparation of the body substance and the body fluid are standard in the art and are described, for example in Manual of Clinical Microbiology, Chapter 8,

"Collection, Handling and Processing of Specimens", 4th edition, Eds, Lennette, E.H., Balows, A., Hausler, W.J. and Shadorny, A.J., American Society for Microbiology, (1986)).

Diagnosis and detection methods also comprise contacting 5 the DNA and RNA of body fluid, tissue, specimen and environmental sample with DNA and RNA of the invention or fragments thereof and the amplification of this specific interaction via PCR, branched chain nucleic acid technology and other amplification technologies such that the presence of 10 *Cryptosporidium* DNA and/or RNA in the bodily fluid, tissue, specimen or environmental sample may be detected. Agents suitable for immunodiagnostic use are proteins comprising epitopes of *Cryptosporidium parvum* that are recognized by intact B and/or T cells. These proteins are produced as 15 described above, purified and used to detect or characterize anti-*Cryptosporidium parvum* antibody in the body substances of populations at risk of prior or current cryptosporidial infection. In addition, antibodies to such proteins are obtained by immunizing animals, such as cows, rabbits or 20 goats, or birds with the vaccine combined with an adjuvant.

Additionally, detections of *Cryptosporidium* may be made by determining cryptopain activity in biological or environmental samples by methods used and known in the art.

25 V. Immunotherapy and Prophylaxis

The immunotherapy of cryptosporidiosis in humans and animals may be conducted by administration of the antibodies of the invention to patients with cryptosporidiosis to effectively reduce their symptomatology.

30 A method for immunotherapeutic treatment, retardation, or inhibition of *Cryptosporidium* infection comprises administering to a subject in need of such treatment an amount of an anti-*Cryptosporidium* polyclonal or monoclonal antibody prepared according to the invention, effective to provide

immunity against the invasion of *Cryptosporidium* or effective to inhibit the existing *Cryptosporidium* infection.

A method of prophylaxis of *Cryptosporidium* infection comprises administering to a subject in need of such treatment 5 a vaccine, as described above, comprising the protein or recombinant protein of this invention capable of endogenous development of inhibitory amount of anti-*Cryptosporidium parvum* antibodies.

Typical immunization is achieved by inoculation of the 10 animal, bird or human host with the antigen protein combined with adjuvant.

For passive immunotherapy when used to passively immunize *Cryptosporidium* infected hosts, the polypeptide is first combined with appropriate adjuvants and used for the 15 immunization of cows or other donor animals to produce antibodies which may be administered to patients with cryptosporidiosis infection, particularly to AIDS patients, and to other immunocompromised hosts. Monoclonal antibodies produced in animals, in humans "humanized" from animal sources 20 and produced through chimeric techniques and other derivative techniques may be used for passive immunotherapy.

When in a therapeutic composition, the antigen protein is combined with appropriate adjuvants and used for the immunization of immunocompetent patients who are at risk for 25 cryptosporidiosis either at the time of immunization or in the future. This group includes, but is not restricted to, HIV positive individuals who are still able to respond to vaccination, animal workers, health care workers, day care center children and their caretakers, and children in the 30 developing world.

VI. Qualitative and Quantitative Detection of
Cryptosporidium-Formulations and Kits

Formulations suitable for the administration of

polypeptides and antibodies such as those described herein are known in the art. Typically, other components stimulatory of immune response as well as fillers, coloring, and the like may be added, such as pharmaceutically acceptable excipient, 5 additives and adjuvants.

For qualitative and quantitative determination of the presence of the *Cryptosporidium* infection and environmental contamination, a kit for the diagnosis/detection of *Cryptosporidium* is used. The kit comprises the polyclonal 10 antibody or antigen of this invention and a means for detecting the complexing of the antibody with antigen. Another such kit comprises DNA/RNA of the invention for use in detecting complementary DNA/RNA of cryptopain. Another such kit comprises PCR primers for amplification of cryptopain 15 sequences and a method of identifying them.

The kit is utilized for the detection of endogenous antibodies/antigens/DNA/RNA produced by a subject that is afflicted with cryptosporidiosis and antigens/DNA/RNA present in the environmental samples. Even at the early stages where 20 the parasite is commencing invasion of a subject's cells, some amount of the *Cryptosporidium* antigen or the specific antibody may be detected in serum. The kit detects either the antigen with the polyclonal antibodies or the presence of the anti-*Cryptosporidium* antibody with the antigen. The 25 complexing immunoreaction is detected by staining, radiography, immunoprecipitation or by any other means used in the art and suitable for these purposes.

In addition to the above, the kits may also comprise a control compounds, anti-antibodies, protein A/G, and the like, 30 suitable for conducting the different assays referred to above.

The current invention provides an effective treatment and prophylaxis against the cryptosporidiosis infection and means

of detection of the parasite and diagnosis of infection.

The following examples describe procedures used to prepare antigens, antibodies, vaccines and kits of the invention. They are illustrative only and any modification 5 using methods known in the art is intended to be included. The following examples are not to be considered in any way limiting.

EXAMPLE 1

Cryptosporidium parvum Parasites

10 This example describes protocol used for isolation of *Cryptosporidium parvum* parasites from which the *Cryptosporidium* antigen was prepared.

Oocysts of the Iowa isolate of *Cryptosporidium parvum* were passaged through neonatal calves, (Pat Madin Pathasan, 15 Pleasant Hill Farms, Idaho). The passaged oocysts were purified and encysted for use in invasion assays. The detailed protocol for purification and encysting is described in *Infect. Immun.*, 61:4079 (1993). The described protocol was used unmodified.

20 For the DNA experiments described herein DNA was purified from 1×10^9 *Cryptosporidium parvum* according to *Mol. Biochen Parasitol.*, 50:105-114, (1992).

EXAMPLE 2

Preparation of a C. parvum Cysteine Proteinase DNA Probe

25 This example describes procedural used for preparation of the *C. parvum* cysteine proteinase DNA probe.

Cysteine proteinases share DNA homology in the regions coding for the active site amino acids involved in proteolysis, specifically conserved C, H and N residues. This 30 was used in choosing an appropriate oligonucleotide pairs to amplify the genomic DNA from Iowa isolate. The most suitable oligonucleotides were found to be those modeled in *Plasmodium vinckeii* cysteine proteinase sequences around the conserved

cysteine (C) and arginine (N) residues as indicated in Figure 4.

Degenerate oligonucleotides for the active C and N sites of the *Plasmodium vinckeii* cysteine proteinase were used to 5 amplify a 459 bp genomic DNA fragment from Iowa isolate DNA. In the degenerate oligonucleotides a "/" indicates that the base pair on either side of the "/" could be included at that location in a triplet encoding an amino acid. (I) indicates inosine which will pair in hybridization reactions in a 10 permissive manner. The oligonucleotides were PC4(sense) consisting of AAA-GGA-TCC-TGC/T-GGI-A/TG/CI-TGC/T-TGG-GCI-TT (SEQ ID NO: 9) encoding a BamHI site and the DNA sequence for C-G-S-C-W-A-F (SEQ ID NO: 13) and PC3 (anti-sense) consisting of the DNA sequence 15 TTT-GAA-TTC-CCA-IG/CA/T-A/GTT-IC/TT/G-IAC/T-IAT-CCA-A/GTA (SEQ ID NO: 10) encoding an Eco RI site and the antisense for a protein sequence. The protein sequence in the sense direction is Y-W-I-V/I-K/R-N-S-W (SEQ ID NO: 14). The restriction sites were not required for the experiments described here. 20 As shown in Figure 4, these oligonucleotides represented a 100% match for the seven amino acid C-G-S-C-W-A-F (SEQ ID NO: 13) sequence of *C. parvum* cryptopain and a 100% match for the five amino acids V-R-N-S-W (SEQ ID NO: 15) within the 25 eight amino acid sequence surrounding the conserved N. These matches were sufficient for PCR amplification purposes.

One hundred nanograms of Iowa isolate DNA was amplified using reagents from GeneAmp (Perkin-Elmer, Foster City, Ca) under the following conditions:

Initial denaturation was 94°C for 2 minutes followed by 30 30 cycles of 94°C for 20 seconds, 40°C for 40 seconds and 72°C for 1 minute.

The 459 bp amplification product was isolated, subcloned in the TA vector (TA Cloning kit, Invitrogen, San Diego, CA)

and sequenced using the di-dioxy technique (Stategene Sequenase II Kit).

Iowa isolate sequence so obtained was found to be homologous to cysteine proteinases of a wide variety of 5 organisms. The sequences of papain and the cysteine proteinase of *P. vinckeii* are shown in Figure 4.

EXAMPLE 3

Isolation, Sequencing and Analysis of a
C. parvum Cysteine proteinase gene

10 This example describes isolation, sequencing and analysis of a *C. parvum* cysteine proteinase gene encoding *C. parvum* antigen.

15 The 459 bp amplification product obtained in Example 2, containing a portion of an Iowa isolate *C. parvum* cysteine proteinase gene was labeled with d-dATP³² using random primers and Klenow fragment. The labeled 459 bp probe was used to screen a NINC, (naturally infected neonatal calf) λgt11 genomic expression library.

20 Three clones, designated E1.6, E4 and RCB1.2, were identified in the library and were purified to homogeneity. Two of them, E1.6 and E4, were subcloned in Bluescript for sequencing (Sequence II kit) and were found to contain the complete cryptopain sequence and 5' and 3' flanking sequences as determined by analysis of the open reading frames within 25 the clones and Genebank Search using the deduced amino acid sequence.

The entire sequence of E1.6 is designated SEQ ID NO: 1 and includes flanking sequences 5' and 3'. The mature enzyme sequence is designated SEQ ID NO: 3.

30 EXAMPLE 4

Southern Hybridization

This example describes Southern hybridization method used to detect the gene of the invention in genomic DNA.

One mg of Iowa DNA was digested with the restriction enzymes, Hind III, Hae III, Nsi, Scr II and combinations thereof, according to conditions for use of each enzyme as provided by the manufacturer (Promega). Digested DNAs were 5 subjected to electrophoresis in 0.8% agarose gels in 1X TAE. The gel was blotted to a nylon membrane Hybond N+, obtained from Amersham per manufacturer's instructions.

Results are seen in Figure 5 which shows a generic Southern analysis using DNA cut and separated in this manner 10 (lane 1=Hind III, lane 2=HaeIII, lane 3=HindIII/HaeIII, lane 4=NsiI, lane 5=SrcII and lane 6=Nsi/SrcII). The 459 bp probe was labeled with ^{32}P -ATP and hybridized to the membrane.

EXAMPLE 5

Preparation of Recombinant Cryptopain

15 This example describes the preparation of recombinant cryptopain.

The primers 7B1 and 7B2 (Figure 7B) were synthesized at the Biomedical Research center, University of California, San Francisco. 7B1 is a sense oligonucleotide and is comprised of 20 a KpnI restriction enzyme recognition site at the 5' end followed by coding sequence for the 5' end of the pre pro cryptopain sequence. 7B2 is an anti-sense oligonucleotide and is comprised of an XbaI sequence at the 5' end followed by the antisense coding sequence of the 3' end of the 25 preprocryptopain sequence. When used as a pair of PCR amplification oligonucleotides, these oligonucleotides allowed the amplification from genomic *Cryptosporidium* DNA of the entire cryptopain gene with Kpn I and Xba I sequences at the 5' and 3' ends respectively.

30 The 7B1 and 7B2 sequences were designed so that after *Kpn*I and *Xba* I digestion of the amplification product, the resultant fragment could be introduced in a directional manner into *pTrxFus* which was cut with *Kpn*I/*Xba*I. Amplified and

restricted DNA was visualized on a 0.8% agarose-1XTAE gel using ethidium bromide. The amplified and endonuclease restricted band was excised from the gel and purified using a glass bead technique (Gene-Clean).

5 pTrxFus was also digested with the enzymes KpnI and Xba I, enzymes uniquely present in the sequence in the poly linker (Figure 8), and the small intervening sequence was removed by gel purification as noted above. pTrxFus and preprocryptopain DNA, prepared in this manner, at 1:1 and 1:5 molar ratios were
10 ligated overnight at 14°C in the presence of ligation buffer and T4 DNA ligase at a concentration of 50-250 ng insert DNA/ 10 μ l.

15 G1724 chemically competent cells were made as described by Xi-Lvitrogen. Three to five μ l of ligation mixes and control mixes were introduced into separate tubes of competent cells and the tubes were incubated on ice for 30 minutes. Tubes were incubated in a 42°C heating block for 90 seconds and placed on ice for 2 minutes. Eight hundred μ l of room temperature of enriched tryptone containing broth medium was
20 added to each tube and the tube was incubated with shaking at 30°C for 60 minutes. Twenty-five and 100 μ l of each transformation mix was plated on RMG-Ampicillin transformation plates and the plates were incubated at 30°C overnight.

25 Nitrocellulose membrane replicas of colonies were prepared from the transformation plates, the adherent cells lysed in alkaline solution and the DNA fixed to the membranes. Nitrocellulose membranes were hybridized with probes to contain cryptopain DNA and following hybridization with a cryptopain, probe were colony purified. DNA was purified from
30 colonies and the identity of the foreign DNA verified by restriction analysis and sequence analysis.

Purified colonies were grown in 1 μ l aliquots for analysis. Growth conditions were varied with respect to time

(2, 3, 4 hours) and the bacteria lysed for evaluation of soluble and insoluble proteins. Results are shown in Figure 9.

Figure 9 shows soluble proteins from 10 μ l of lysate at 5 2, 3, 4 hours in lanes 2, 3, and 4 on an immunoblot of an SDS-PAGE gel. Cryptopain fused to thioredoxin appears as a 57 kDa protein which is appropriate for the size of the fusion partner (12 kDa) and the size of preprocryptopain (35 kDa). Lane 1 is the thioredoxin control. All lanes are visualized 10 with anti-thioredoxin antibody followed by chemiluminescent detection (Amersham). Yield, using this expression system, was maximal at 3 hours of bacterial growth and was estimated at 0.9 mg cryptopain-thioredoxin per 250 ml culture. Although the yield was very high in this system, purification after 15 enterokinase removal of the fusion partner was less satisfactory.

EXAMPLE 6

Large Scale Purification of Recombinant Cryptopain

This example describes the purification procedure for 20 cryptopain.

In order to provide large quantities of cryptopain purified from its fusion partner, thioredoxin, the KpnI/XbaI preprocryptopain DNA fragment of Example 5 was cloned into an improved vector known as pThio His (Invitrogen). The 25 improvements of the invitrogen system were:

- 1) Metal binding sites were engineered into the sequence between the thioredoxin reading frame and the enterokinase recognition site facilitating large scale purification of the fusion protein over chromatography columns (Pro-bond, 30 Invitrogen).
- 2) Growth of transformed bacteria (Top 10, Invitrogen) in the presence of more standard media.
- 3) Ability to cleave the foreign protein from the fusion

partner using enterokinase while the fusion protein was on the nickel column allowing a high degree of purification from the fusion partner.

Colonies were prepared as in Example 5 using Top 10 *E. coli*. Large scale group was accomplished, the bacteria harvested and lysed and the fusion protein collected by passage over Probond or other metal chelation columns. The columns were washed with normal saline and cryptopain was collected by passing dilute enterokinase over the column.

10

EXAMPLE 7

Inhibition of *Cryptosporidium* Invasion and Intracellular Development in MDCK cells with Inhibitors of Cathepsin-L Like Cysteine Proteinases

This example describes studies performed to detect inhibition of *Cryptosporidium* invasion and intracellular development *in vitro* using cathepsin-L-cysteine proteinase inhibitors.

Cryptosporidium oocysts of the Iowa isolate were encysted according to Example 1. To assess the effect of inhibitors E64, BPAFMK and KIII on sporozoite invasion, inhibitors were incubated with viable sporozoites for 30 minutes prior to addition to monolayers of MDCK cells as described in (J. Protozool., 386:556 (1991); and Infect. Immunol., 61:4079 (1993).

25 Sporozoite invasion and intracellular development in MDCK cells was scored at 16 hours after fixation of MDCK cells in formalin and staining with Giemsa.

EXAMPLE 8

30 Detection of proteinase activity as a measure of viability of *Cryptosporidium* organisms

This example describes a method for detection of proteinase activity as a measure for viability of *Cryptosporidium* organism in environmental samples.

Cryptosporidium cannot be grown in culture *in vitro*. Available evidence indicates that acquisition of cryptosporidiosis from water, food and other environmental sites is a major source of disease spread. However, reliable 5 methods of determining whether living *Cryptosporidium* species are present in a sample have not been developed.

The invention provides a method assaying activity of proteins which have a short half-life. Proteinases which are tightly regulated with respect to activation, because 10 unrestricted activity would damage the integrity of the cell, represent one such type of proteins.

Highly specific active site inhibitors of cryptopain are used for evaluation of viability of *Cryptosporidium* organisms. A highly specific inhibitor of cryptopain, for example E64, 15 KIII or pre pro cryptopain protein is labeled with a radioactive, chemiluminescent, colorimetric or other tag. The tagged inhibitor is incubated with *Cryptosporidium* organisms/proteins from an environmental sample and the amount of tag bound/organism relative to positive and negative 20 control is ascertained. Number of organisms may be determined by flow cytometry.

EXAMPLE 9

Agents Suitable for Passive Immunotherapy

This example describes preparation of suitable agents for 25 passive immuno therapy.

Recombinant cryptopain described in Example 5, or a recombinant fragment of cryptopain with or without fusion protein are used to immunize animals such as cows, goats or rabbits. The antibody developed in the body of the animal is 30 purified from serum or milk as colostrum or used without purification for treatment of a *Cryptosporidium* infection of mucosal surfaces.

The antibody is delivered orally or through a tube and is

optionally mixed with agents or substances which delay or prevent the inactivation of antibody in the gastrointestinal tract.

EXAMPLE 10

5 Agents Suitable for Active Immunotherapy

This example illustrates agents derived from *C. parvum* suitable for active immunotherapy.

10 Recombinant cryptopain according to Example 5, or recombinant fragments of cryptopain with or without fusion protein is used to immunize animals or humans in such a way that the animal or human develops antibody or cell mediated immune responses to *Cryptosporidium* which ameliorate or inhibit infection by *Cryptosporidium*.

EXAMPLE 11

15 Agents Suitable for Immunodiagnostic/Immunodetection Use

This example illustrates procedure for obtaining agents derived from *Cryptosporidium parvum* for suitable immunodiagnostic/immunodetection use.

20 Recombinant cryptopain or recombinant fragments of cryptopain or antibodies (monoclonal, polyclonal or chimeric) raised to recombinant cryptopain or recombinant fragments of cryptopain are used to detect the corresponding antibody or antigen in a soluble or fixed assay.

25 Recombinant cryptopain is immobilized in wells and utilized to detect the corresponding antibody from humans or animals by capture of the antibody and colorimetric or other detection method.

30 Correspondingly, antibodies to recombinant cryptopain are immobilized in wells and utilized to detect cryptopain in secretions or feces or other bodily fluids or environmental samples. Both of these assays are also be performed in a soluble format.

EXAMPLE 12

Detection of mRNA as a Measure of Viability
of Cryptosporidium Organisms

This example illustrates detection of mRNA as a measure of viability of *Cryptosporidium* organisms.

5 The presence of mRNAs which have a short half-life was assayed on the basis of the fact that many mRNAs are destroyed within 2 minutes of production and the amount of intact mRNA in a cell provides a measure of the viability of an organism.

10 A probe for hybridization with the mRNA of the invention is prepared and labelled with radioactive, chemiluminescent, colorimetric or other tag. The tagged probe is incubated with *Cryptosporidium* organisms from an environmental sample and the amount of tag bound/cell relative to positive and negative controls is ascertained. Number of organisms is determined by 15 flow cytometry or any other suitable means.

EXAMPLE 13

Agents Suitable For Nucleotide Based Diagnosis/Detection

This example illustrates the procedure for obtainin agents derived from *C. parvum* for nucleotides based diagnosi 20 and/or detection.

Oligonucleotides or PCR amplification products using nucleotides derived from the cryptopain or the flanking DNA sequences is used to detect *Cryptosporidium* in human or animal samples or in the environment.

25 Oligonucleotides are used to amplify a *Cryptosporidium* fragment as described in the Examples above from the samples or from the environment and to detect its presence in either location. PCR amplification products or segments of DNA or RNA are used as probes to detect the presence in either 30 location in hybridization experiments. Hybridization is either as a Southern blot or as a dot blot. The hybridization signal is amplified by a variety of techniques including the branched chain technique.

PCT Definition

EXAMPLE 14Preparation of Anti-*Cryptosporidium* Vaccines

This example describes preparation of anti-*Cryptosporidium* vaccines using DNA, RNA or amino acid 5 cryptopain sequences.

A vaccine for prevention and treatment of infections caused by protozoan *Cryptosporidium* species (*Cryptosporidium*) in humans and other mammals was developed by utilizing newly identified and isolated DNA and amino acid sequences of the 10 *Cryptosporidium* pathogen designated cryptopain.

The antigen proteins used for preparation of vaccines correspond to cryptopain (SEQ ID NO: 4) which is identified by being a target of the polyclonal or monoclonal antibodies of the invention capable of preventing or ameliorating disease 15 and preventing invasion and/or intracellular development in host cells.

A DNA or RNA vaccine for prevention and treatment of infections caused by protozoan *Cryptosporidium* species (*Cryptosporidium*) in humans and other mammals was developed by 20 utilizing newly identified and isolated DNA (SEQ ID NOS: 1-3) and amino acid sequences of the *Cryptosporidium* pathogen designated cryptopain.

A hybrid vector comprising a DNA segment that encodes the protein antigen able to bind selectively and specifically to 25 anti-*Cryptosporidium* antibodies operatively coupled to the vector was prepared and expressed as described in Example 5. This includes preparation of recombinant vaccines using the viral expression vector according to Example 5 outside of the host body but also includes preparation of DNA vaccines and 30 procaryotic or eukaryotic host carrying the hybrid vector which may be introduced into the host vertebrate or a direct introduction of DNA or RNA into host cells generating the hosts own expression or translation of DNA or RNA to produce

proteins eliciting appropriate antibodies.

EXAMPLE 15

Preparation of Anti-Cryptosporidium parvum Vaccine

This example illustrates procedure for preparation of
5 anti-Cryptosporidium parvum vaccine of the invention and its
use.

Vaccines use of recombinant *Cryptosporidium* antigens
prepared according to Examples 5 and 14.

(1) Antigens

10 Preferably 10-200 μ g of recombinant antigen of the
invention, either alone or in combination is sued for
preparation of the vaccine.

(2) Adjuvant

15 Any one of a number of adjuvants designed to either:

(a) stimulate mucosal immunity; or
(b) target mucosal lymphoid tissue is sued for
preparation of the vaccine of the invention.

20 Examples of these adjuvants are: liposomes, saponins,
lectins, cholera toxin B subunit, E. coli labile toxin (LT) B
subunit, pluronic block copolymers, hydroxyapatite, plant
glucans, acetyl mannan (from Aloe Vera), aluminum hydroxide.

(3) Route of administration

Since the vaccine must stimulate mucosal immunity, it
preferably is delivered to a mucosal site.

25 Feasible routes of administration include: oral, nasal,
rectal, and vaginal. However, boosting may occur via another
route.

(4) Volume

30 The volume of the vaccine, while not particularly
important, should be in the range that would permit ease of
use. Preferred range would be about 0.5 ml-2.5 ml, including
adjuvant, per one vaccine dose.

(5) Boost schedule

Since this vaccine would be intended for immunocompromised individuals, one would expect the diminishing immune status to require a more aggressive boosting schedule than would otherwise be necessary.

5 The vaccine is administered to high risk patients initially when their immune status is reasonably good (i.e., CD4 count of >500). Booster schedules are typically given initially at 1 month after the primary immunization, and again every 3-4 months during progression of the immunodeficient
10 state.